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ORIGINAL ARTICLE

Characterization of a histidine- and alanine-rich protein showing interaction with calreticulin in rice

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Abstract Calreticulin (CRT) is a major calcium-sequestering protein in the endoplasmic reticulum and has been implicated in a variety of cellular functions. To analyze the function of CRT in rice, a yeast two-hybrid protein interaction assay was used for identifying interacting proteins. Fourteen of 17 interacting cDNA clones found coded for a novel histidine- and alanine-rich protein (OsHARP) of 342 amino acid residues. The mRNA expression level of OsHARP was up-regulated in rice seedlings treated with gibberellin (GA), but not ABA and showed a similar pattern as OsCRT mRNA. Rice plants transformed with the OsHARP promoter-GUS construct showed GUS staining in the basal parts of leaf sheaths, and although GUS activity increased when treated with GA_3, it was not as high an increase as when mRNA was analyzed. To elucidate the role of OsHARP in leaf sheath elongation, antisense OsHARP transgenic rice lines were constructed. The antisense OsHARP transgenic rice plants were consistently shorter than the vector control under normal conditions. To examine whether OsHARP expression would affect other proteins, basal leaf sheaths from antisense OsHARP transgenic rice plants were analyzed using proteomic techniques. In antisense transgenic-rice OsHARP plants, OsCRT was down-regulated and the levels of 20 other proteins were changed compared to the pattern of the vector control. These results signify an important role of HARP in rice leaf sheath cell division or elongation and suggest that CRT may interact with HARP during certain stages of development.

Keywords Calreticulin · Histidine- and alanine-rich protein · Leaf sheath elongation · Rice

Abbreviations

HARP	Histidine- and alanine-rich protein
CRT	Calreticulin
GA	Gibberellin
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
CBB	Coomassie brilliant blue
PBS	Phosphate-buffered saline
PVDF	Polyvinylidene difluoride
IEF	Isoelectric focusing
MS	Mass spectrometer
BL	Brassinolide
BA	6-Benzyladenine
2,4-D	2,4-Dichlorophenoxyacetic acid
ABA	Abscisic acid

Introduction

Calcium is an essential second messenger playing an important role in regulating a wide range of developmental, physiological and fundamental cellular processes in plants and animals (Clapham 1995; Corbett and Michalak 2000). Changes in cytoplasmic calcium ion concentration as functions of time and location that are produced by a particular signal are referred to as a calcium signature (Scrase-Field and Knight 2003). Although such calcium

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signatures may partially explain the specificity of cellular responses triggered by a particular stimulus, the molecules that "sense" and "interpret" the calcium signals provide additional specificity to the coupling of calcium parameters to cellular responses (McAinsh et al. 1992). Several families of calcium sensors have been identified in higher plants and are broadly divided into four major classes: calmodulin, calmodulin-like and other EF hand-containing calcium-binding proteins, calcium-regulated protein kinase and calcium-binding proteins without an EF-hand motif such as calreticulin (CRT) (Zielinski 1998; Harmon et al. 2000; Crofts and Denecke 1998; Liewelyn et al. 1998).

CRT is a major calcium-sequestering protein found in the lumen of the endoplasmic reticulum of a wide variety of eukaryotic cells, including those of higher plants (Corbett et al. 1999; Opas et al. 1996). In plants, CRT homologues have been purified and sequenced from spinach (Menegazzi et al. 1993), tobacco (Denecke et al. 1995), pea (Hassan et al. 1995), maize (Kwiatkowski et al. 1995) and rice (Komatsu et al. 1996; Li and Komatsu 2000). Abundant accumulation of CRT has been observed during callus regeneration in rice (Komatsu et al. 1996), during tobacco germination (Denecke et al. 1995), and in most plant tissues such as roots, young leaves (Menegazzi et al. 1993), and floral tissues of Arabidopsis (Nelson et al. 1997). Furthermore, Persson et al. (2003) reported the presence of two distinct CRT isoform groups, with distinct expression patterns and posttranslational modifications, supporting functional specificity among plant CRTs and possibly accounting for the multiple functions assigned to CRTs.

In rice, OsCRT has been identified as a calcium-binding phosphorylated protein that appears to be associated with the regeneration of cultured rice cells (Komatsu et al. 1996). OsCRT is also present in rice suspension culture cells, and is developmentally regulated during regeneration (Li and Komatsu 2000) and with cell-cycle-related variation (Takase et al. 2003). OsCRT has been shown to be involved with (Imin et al. 2006) and phosphorylated in the signaling pathway leading to cold stress response (Li et al. 2003; Khan et al. 2005). To investigate the biological role of OsCRT in rice, OsCRT interacting protein 1 (OsCRTintP1) was isolated by a yeast two-hybrid interaction-cloning system (Sharma et al. 2004). Sharma et al. (2004) reported that co-immunoprecipitation using an anti-OsCRT antibody confirmed the existence of the OsCRT–OsCRTintP1 complex in vivo in cold-stressed leaf tissue. Furthermore, over-expression of calcium-dependent protein kinase 13 and *OsCRTintP1* conferred cold tolerance on rice plants (Komatsu et al. 2007).

Additionally, Shen et al. (2003) and Komatsu et al. (2006) reported that OsCRT was also an important component in the gibberellin (GA) signaling pathway that regulates leaf-sheath elongation in rice seedling; however, the role of OsCRT in rice leaf sheath elongation is not clear. The present study was designed to identify novel proteins that interact with OsCRT in regulating GA signaling processes in rice by using a yeast two-hybrid interaction-cloning system. When a full-length cDNA of OsCRT was used as bait to screen a cDNA library prepared from the basal parts of rice leaf sheaths, a histidine- and alanine-rich protein (OsHARP) was cloned and characterized.

Materials and methods

Plant materials and treatments

Rice (*Oryza sativa* L. cv Nipponbare) was grown under white fluorescent light (600 μmol/m^2 per s, 12 h light period/day) at 28°C and 75% relative humidity in a growth chamber. Experiments were conducted in plastic seedling pots (280 × 160 × 90 mm) in growth chamber. For hormone experiments at 2 weeks after sowing, seedlings were treated with GA_3 (Wako Pure Chemical, Osaka, Japan), brassinolide (BL, Fuji Chemical, Toyama, Japan), 6-benzyladenine (BA, Wako Pure Chemical), 2,4-dichlorophenoxyacetic acid (2,4-D, Wako Pure Chemical) and abscisic acid (ABA, Wako Pure Chemical) for 24 h. Phytohormone stock solutions were made with dimethyl sulfoxide and control treatment contains the same amount of dimethyl sulfoxide (final concentration 0.1% v/v).

Yeast two-hybrid assay

The BD Matcher™ library construction and Screening kits (Clontech, Palo Alto, CA, USA) was used to screen for the in vitro interaction between OsCRT and proteins obtained from cDNA library prepared from rice leaf sheath including basal part of leaf sheath. All procedures were performed according to the manufacturer's protocol (Clontech). Briefly, full-length *OsCRT* cDNA was fused in-frame with GAL4 DNA binding domain in the pGBKT7 vector. cDNA was cloned into pGADT7 vector encoding the GAL4 activation domain. The GAL 4 fusion constructs were used for the transformation of yeast strain AH109 and the cells were plated on synthetic medium. Screening for the protein–protein interaction events was performed according to the manufacturer's instructions (Clontech).

Preparation of anti-OsHARP antibody

Recombinant OsHARP was prepared according to the manufacture's instructions (Novagen, Madison, WI, USA).

Recombinant OsHARP protein (1 mg) was column purified and electroeluted. The protein solution was then dialyzed against deionized water for 2 days and dried. Using this protein, an antibody against OsHARP was raised in adult rabbit (Bailey 1985).

Co-immunoprecipitation

Total protein from basal part of leaf sheath was extracted, and incubated in phosphate-buffered saline (PBS) with either anti-OsCRT or anti-OsHARP antibodies for overnight at 4°C. Pre-immune antisera were used as negative controls. Protein A-agarose (Sigma-Aldrich, St Louis, MO, USA) beads were added and incubation was continued for 4 h at 4°C. Pellets were washed by centrifugation six times in PBS. After final wash, pellets were re-suspended in 100 µL SDS-sample buffer (Laemmli 1974) and boiled for 3 min. The proteins present in the immune complexes were separated on SDS–polyacrylamide gel electrophoresis (PAGE) gel for Western blot.

Protein extraction and Western blot analysis

Proteins were separated by SDS–PAGE and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Pall, PortWashington, NY, USA) followed by Western blot analysis with an antibody or Coomassie brilliant blue (CBB) staining. For Western blot, the PVDF membranes were blocked with 3% gelatin in TBS containing 20 mM Tris–HCl (pH 7.5), 500 mM NaCl. The anti-OsHARP antibody and anti-OsCRT antibody (Komatsu et al. 1996) were used at working solution of 1:1000 with 16 h incubation. After washing, anti-rabbit IgG horseradish peroxidase (HRP; Bio-Rad, Hercules, CA, USA) diluted to 1–3,000 was used as an indirect label for 1 h at room temperature. The PVDF membrane was stained by the HRP color development reagent (Konica-Minolta, Tokyo, Japan).

RNA extraction and Northern blot analysis

Tissue samples were quick-frozen in liquid nitrogen. Samples were ground to powder using a mortar and pestle, and total RNAs were isolated according to the procedure described by Chomczynski and Sacchi (1987). For Northern blot analysis, 20 µg of total RNA was separated on 1.2% agarose containing 6% formaldehyde and transferred onto Hybond^{TM}-N^{+} nylon membrane (GE healthcare, Piscataway, NJ, USA). Loading of equal amounts of total RNA for Northern blots was determined by visualization of ethidium bromide-stained rRNA bands. The probes for *OsHARP* and *OsCRT* were full-length cDNA. PCR products were purified from agarose gel (Qiagen, Hilden, Germany), and radio labeled using [α-^{32}P] dCTP (GE healthcare) and the random prime labeling system (Rediprime^{TM} II, GE healthcare). Hybridization was performed at 42°C in an ultrasensitive hybridization buffer (ULTRAhyb^{TM}, Ambion, Austin, TX, USA) overnight. The blots were washed twice in 2× SSC, 0.1% SDS at 42°C for 5 min, and in 0.1× SSC, 0.1% SDS at 68°C for 15 min, and analyzed by phosphor image program using Typhoon 8,600 k variable imager (GE healthcare).

Cloning of OsHARP promoter fragment and GUS localization

Two thousand base pairs long *OsHARP* promoter fragment was PCR amplified from rice genomic DNA with primer pairs using KOD plus (Toyobo, Osaka, Japan) using the PCR conditions; 94°C for 2 min (1 cycle), 94°C for 15 sec, 63°C for 30 sec, 68 °C for 2 min (30 cycles), 68°C for 7 min (1 cycle). The PCR product was purified from agarose gel (Qiagen) and cloned into Gateway entry vector pENTR/SD-TOPO (Life technologies, Carlsbad, CA, USA) and then into binary vector pHGWFS7 through LR recombination (Karimi et al. 2002). The resulting vector carrying *OsHARP* promoter::GUS fusion was transformed into rice via *Agrobacterium*-mediated transformation.

GUS staining was conducted according to Jefferson (1997). Seedlings of transgenic plants were incubated in 50 mM sodium phosphate buffer (pH 7.2) containing 1.0 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Wako Pure Chemical) and 5% methanol at 37°C for 24 h. The reaction was stopped by adding ethanol. Two-week-old *OsHARP promoter::GUS* transgenic rice seedlings were treated without or with 1 and 10 µM GA_3 for 24 h. Plants were homogenized in extraction buffer containing 50 mM sodium phosphate (pH 7.0), 10 mM EDTA, 10 mM β-mercaptoethanol, 0.1% (v/v) sodium n-lauroyl-sarcosine and 0.1% (v/v) Triton X-100. To assay GUS activity, 150 µL of extract was mixed with 200 µL of 1 mM 4-methylumbelliferyl β-D-glucuronide in extraction buffer prewarmed to 37°C. The mixture was incubated at 37°C for 30 min, and 100 µL mixtures was mixed with 2 mL of stop solution (0.2 M sodium carbonate). The resulting fluorescence was measured with MTP-100F microplate reader (Corona Electric, Ibaraki, Japan) at 365 (excitation) and 465 (emission) nm. Protein content was determined according to Bradford method (1976) to normalize GUS activity.

Construction of OsHARP transgenic rice

For OsHARP over expression and antisense transgenic rice, the full-length cDNA sequences in the pBluescript SK + plasmids were amplified by PCR using primer pairs. The resulting PCR products were digested, purified, and ligated between the 35SCaMV promoter and nopaline synthase terminator in the binary vector pIG121-Hm by replacing the GUS coding region (Ohta et al. 1990). The pIG121-Hm/OsHARP constructs were confirmed by restriction mapping and sequencing. The pIG121-Hm/OsHARP plasmids and vector control were then transferred into *Agrobacterium tumefacien* strain EHA105 (Hood et al. 1986) and transformed into rice (Toki 1997). Transgenic rice plants were selected on medium-containing hygromycin. The hygromycin-resistant rice plants were transplanted to soil and grown to maturity at 30°C in 16 h light/8 h dark cycle in an isolating greenhouse.

Protein extraction and two-dimensional polyacrylamide gel electrophoresis

Samples (200, 100 µl) solubilized with lysis buffer containing 8 M urea, 2% Triton X-100, 2% ampholine (pH 3.5–10) and 10% polyvinylpyrrolidone-40 (O'Farrell 1975) were separated in the first dimension by isoelectric focusing (IEF) tube gel and in the second dimension by SDS–PAGE. IEF tube gel solution consisted of 8 M urea, 3.5% acrylamide, 2% NP-40, 2% ampholines (pH 3.5–10.0 and 5.0–8.0), ammonium persulfate and TEMED. Electrophoresis was carried out at 200 V for 30 min, followed by 400 V for 16 h and 600 V for 1 h. After IEF, SDS–PAGE in the second dimension was performed using 15% polyacrylamide gel. The gels were stained with silver or CBB, and the image analysis was performed. Images of two-dimensional (2D) -PAGE were evaluated automatically using ImageMaster 2D Elite software (GE Healthcare). The pI and Mr of each protein were determined using 2D-PAGE markers (Bio-Rad).

Amino acid sequence analysis

Following separation by 2D-PAGE, the proteins were electroblotted onto a PVDF membrane using a semidry transfer blotter (Nippon Eido, Tokyo, Japan), and detected by CBB staining. The stained protein spots were excised from the PVDF membrane and applied to a gas-phase protein sequencer Procise 494 (Applied Biosystems, Foster City, CA, USA). The amino acid sequences obtained were compared with those of known proteins in the Swiss-Prot, PIR, Genpept and PDB databases with Web-accessible search program FastA.

Electrospray ionization quadrupole time of flight mass spectrometric analysis

The CBB stained protein spots were excised from gels and destained with 50 mM NH_4HCO_3 in 50% methanol at 40°C for 15 min. Proteins were reduced with 10 mM DTT in 100 mM NH_4HCO_3 at 50°C for 1 h and incubated with 40 mM iodoacetamide in 100 mM NH_4HCO_3 for 30 min. The gel pieces were minced and allowed to dry and then rehydrated in 10 mM Tris–HCl (pH 8.5) with 1 pmol trypsin at 37°C for 10 h. The digested peptides were extracted from the gel slices with 0.1% trifluoroacetic acid in 50% acetonitrile/water for three times. Peptides were analyzed with a hybrid quadrupole orthogonal acceleration tandem mass spectrometer (Q-TOF MS; Micromass, Manchester, UK) connected with LC (Waters, Milford, MA, USA). MS/MS data were processed with a maximum entropy data enhancement program MaxEnt 3^{TM} (Micromass). The resultant spectra were interpreted with SeqMS, software aids for de novo sequencing by MS/MS. The sequence tags obtained were also used for the homology search in the database with Mascot software (Matrix Science Ltd., London, UK).

Results and discussion

A histidine- and alanine-rich protein interacts with calreticulin in basal parts of rice leaf sheaths

To analyze the function of OsCRT, a yeast two-hybrid interaction cloning system was used for identifying interacting proteins. A full-length OsCRT cDNA was used as a bait to screen a cDNA library prepared from basal parts of rice leaf sheaths containing the meristem. Yeast colonies that were positive for all three-reporter genes were selected as candidates for true interactions. A total of 17 positive cDNA clones were verified to be truly positive upon re-examination by yeast-hybrid assays (Table 1). These cDNAs were sequenced and subjected to a homology search. Fourteen clones out of 17 interacting cDNAs showed identical nucleotide sequences but were heterogeneous in their length. To further confirm the specificity of these clones, an unrelated protein, RuBisCO activase, in place of OsCRT was used as a bait to test the interaction with basal parts of leaf sheath cDNA library proteins. No positive clones identical to the 14 OsCRT interacting cDNA clones were obtained. This result indicates that the

Table 1 Screening of OsCRT-interacting proteins in yeast two-hybrid assay

	RuBisCO activase (control)	OsCRT#1	OsCRT#2
OsHARP	0	9	5
Others	12	3	0
Total	12	12	5

RuBisCO activase was used as a negative control and the experiment was repeated twice using OsCRT as a bait

cloned gene corresponding to the 14 OsCRT interacting cDNAs is not an artifact.

The complete sequences of the interacting cDNA clones were obtained by a 5'RACE strategy. Sequence analysis of the full-length amplified fragment revealed a complete open reading frame. The interacting protein consists of 1,082 nucleotides and codes for a novel protein of 342 amino acid residues, with a predicted pI of 5.94 and molecular weight of 39,769 Da (Accession number AK101438). The primary sequence contains an unusually high content of histidine (14%), glutamic acid (14%) and alanine (13%) residues (Fig. 1). This protein was designated as a histidine- and alanine-rich protein (OsHARP). No sequence homology of OsHARP with any known functional protein was found in the existing database.

To understand the biological significance of OsCRT and OsHARP interaction as detected by two-hybrid analysis, in vivo interaction between the two native proteins was evaluated by a co-immunoprecipitation assay (Fig 2). Recombinant OsHARP was purified and electroeluted, and used to raise an antibody against OsHARP in adult rabbits. Total soluble protein from basal parts of rice leaf sheaths was used for co-immunoprecipitation. Rabbit preimmune serum (control) was immunoprecipitated with an anti-OsCRT antibody (Komatsu et al. 1996) or the anti-OsHARP antibody. Immunoprecipitated proteins were cross-reacted with anti-OsHARP antibody (Fig. 2 left) and anti-OsCRT antibody (Fig. 2 right). After immunoprecipitation with anti-OsCRT antibody, two OsHARP bands were detected by anti-OsHARP antibody, and two OsCRT were detected

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001 MARLAVAAALLLVALCRVVHGEADQETAPAYANREAYVEHNGGDAAGTSV 050
051 AIVPPYLDAAAAARDEPAAAAASPEGPVIPVDDDAADQQGFLRFPCRYHC 100
101 RYRHHMRHGGHRHGEGFHGKEEKQQLVFEMPVEPATRGEERREEEEGVVL 150
151 PVAEPDPDSRRQYAAVAAAEEEDEDEMARLHHGRRSHHHHHRHHHHHHDE 200
201 NEEDEHEQADEASPAVERLISFHRRRHHHHHHEDDHEQREEGAPMKRFRH 250
251 HHEEEEESEMRTKRFHHHHHKDDDERELEEMARRWIRKALMSSRMHHHRG 300
301 CRFHHHHHHLSFRHRAEDAAAAGEEEEKGGVMSWLKDFVNRF 342
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Fig. 1 The primary sequence of OsCRT interacting protein, a histidine- and alanine-rich protein, OsHARP. The *underlined sequence* shows alanine-rich and histidine-rich regions

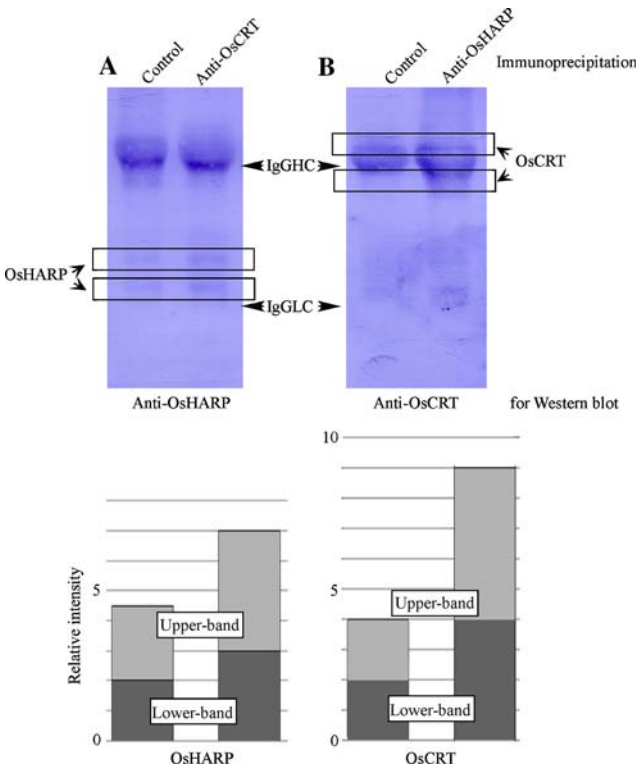

Fig. 2 Co-immunoprecipitation assay showing in vivo interactions between OsCRT and OsCRT interacting protein, OsHARP. Antibodies for OsCRT and OsHARP were prepared from the protein purified from 2D-PAGE gels and the over-expressed protein in *E. coli*, respectively. After immunoprecipitation with anti-OsCRT antibody (**a**) or anti-OsHARP antibody (**b**), precipitates were separated by SDS–PAGE, transferred onto PVDF membrane, and reacted with anti-OsHARP antibody (**a**) or anti-OsCRT antibody (**b**). *Boxes* show the positions of OsHARP and OsCRT. *Bar* diagram shows the relative intensity, which is the band volume in *top panel*, of OsHARP and OsCRT bands. The values are the means of three independent experiments

by anti-OsCRT antibody after immunoprecipitation with anti-OsHARP antibody. These results indicate that OsCRT and OsHARP interacted. In rice, because there are two OsCRT (Li and Komatsu 2000), immunoprecipitation with OsHARP might be detected two OsCRT. However, the reason of two bands of OsHARP detected with OsCRT is not understood.

Pandey et al. (2001) has reported that histidine-rich protein II was a naturally occurring histidine- and alanine-rich protein localized in several cell compartments including the cytoplasm. Their studies have implicated histidine-rich protein II as an important factor in the detoxication of heme (Pandey et al. 2001). The production of histidine-rich protein II was closely associated with the development and proliferation of the malarial parasite and, therefore, was perfectly suited to reflect growth inhibition as a measure of drug sensitivity (Noedle et al. 2002). At the present time, however, the function of histidine- and alanine-rich proteins is not known in plants.

OsHARP is up-regulated in basal parts of leaf sheath by gibberellins

To study the hormonal expression pattern of OsHARP mRNA, its expression was analyzed in response to different hormones (Fig. 3). The basal parts of leaf sheaths were treated with 5 μM each GA_3, BA, 2,4-D, ABA and 1 μM BL for 24 h. The expression of OsHARP mRNA was up-regulated by GA_3 and BL treatment, and down-regulated by BA, 2,4-D and ABA treatment. Furthermore, GA_3 remarkably up-regulated and ABA down-regulated the expression of *OsCRT* (Fig. 3). GA induces internodal elongation, and the initial growth response is due to increased cell elongation (van der Knaap et al. 2000). This result suggests that OsHARP might be actively involved in early GA-induced cell elongation processes. Moons et al. (1995) reported that a 40 kDa histidine-rich protein is found in rice plants in which shoot growth was inhibited by ABA and the proteins is up-regulated by ABA treatment. Based on its response to ABA, OsHARP expression appears to be distinct from this previously reported histidine-rich protein.

Several groups have shown that different GA up-regulated genes are expressed in an organ-specific manner (Ben-Nissan et al. 2004; Jan et al. 2006a). In order to examine the organ-specific expression of OsHARP, proteins from rice root, the basal part of leaf sheaths, leaf sheaths and leaf blades were cross-reacted with anti-OsHARP antibody (Fig. 4). A strong signal was detected in the basal part of leaf sheaths, but not in leaf blades. The *OsHARP* 5′-upstream region, with a length of 2.0 kbp, was fused to GUS in a binary vector and expressed in rice (Fig. 5a). Histochemical localization of GUS activity was detected in the basal part of leaf sheaths and coleoptiles in young seedlings. To examine whether the promoter region of *OsHARP* was capable of hormonally regulating *OsHARP* expression, rice plants were treated with 1 and 10 μM GA_3 for 24 h (Fig. 5b). GUS activity was measured by the fluorogenic method using MUG as a substrate. Transgenic plants treated 10 μM for 24 h had almost 1.5 fold higher GUS activity compared to untreated plants. These results indicate that the 2.0 kbp promoter region of *OsHARP* is sufficient to up-regulate the GUS reporter gene in response to GA_3.

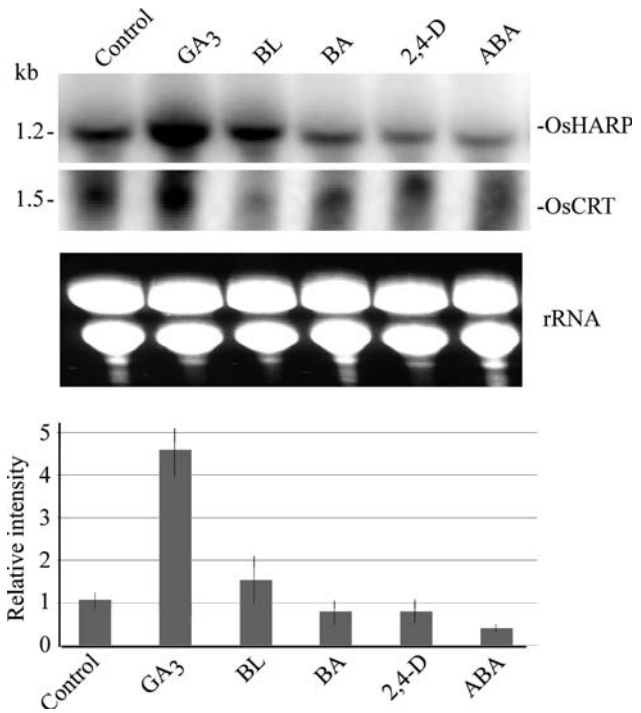

Fig. 3 Effect of phytohormones on *OsHARP* and *OsCRT* mRNA expression. Two-week-old rice seedlings were treated with 1 μM BL, or 5 μM each GA_3, BA, 2,4-D, or ABA for 1 day. Total RNAs were extracted from basal parts of leaf sheaths, and probed with full-length cDNA of OsHARP or OsCRT. rRNA stained with ethidium bromide was used a loading control. *Bar* diagram shows the relative intensity, which is the band volume in *top panel*, of OsHARP bands. The values are the means and ± SE of three independent experiments

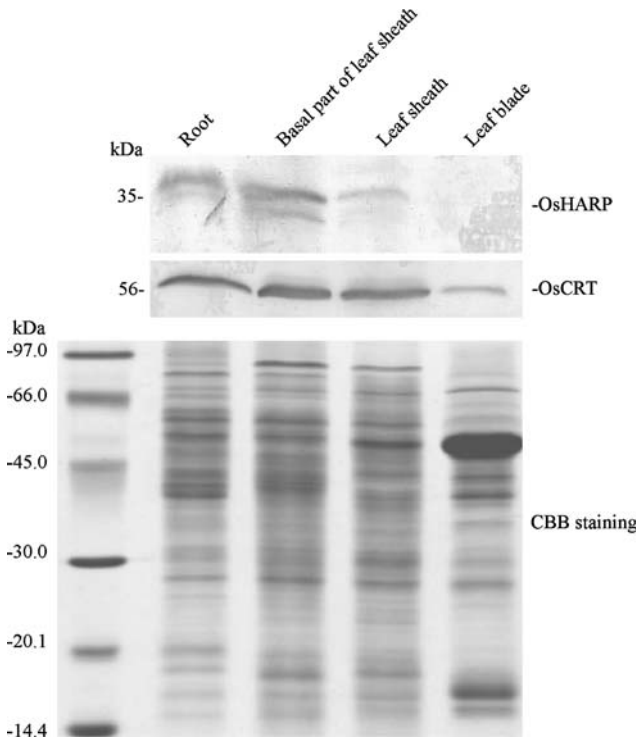

Fig. 4 Organ-specific expression of OsHARP and OsCRT protein. Root, basal part of leaf sheaths, leaf sheaths and leaf blades were cut from two-week old seedlings. Proteins (15 μg) were extracted, separated by SDS–PAGE and transferred onto PVDF membrane. Proteins were reacted with anti-OsHARP antibody or anti-OsCRT antibody. Proteins stained with CBB were used as a loading control

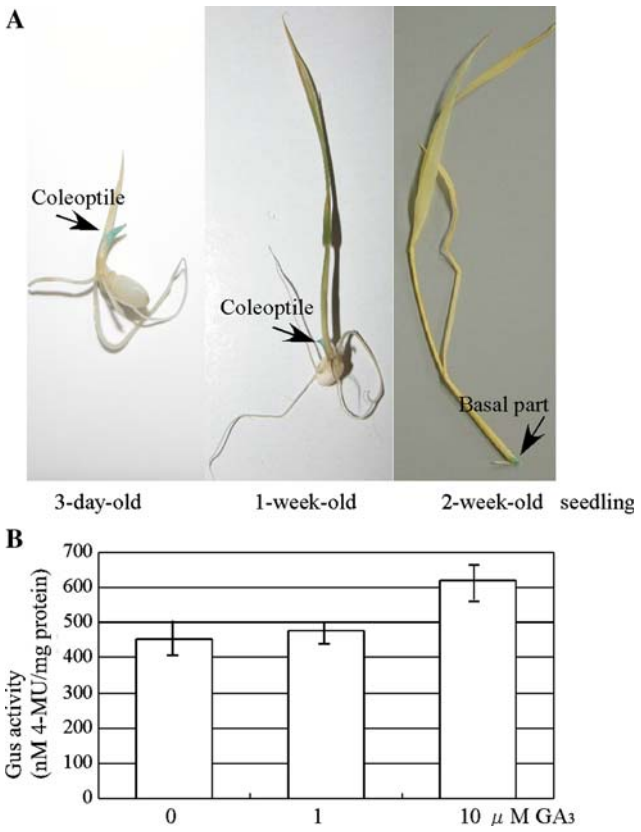

Fig. 5 GUS activity in *OsHARP::GUS* transgenic rice seedlings in response to GA_3 treatment. Three-day-old, one-week-old and two-week-old transgenic rice seedlings were stained in GUS staining buffer containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (**a**), two-week-old seedlings were treated with 1 and 10 μM GA_3 for 24 h. Gus activity was measured by a fluorescent method as described in "Materials and methods" (**b**). The values are the means and ± SE of five plants, and the assay was done twice using different transgenic lines

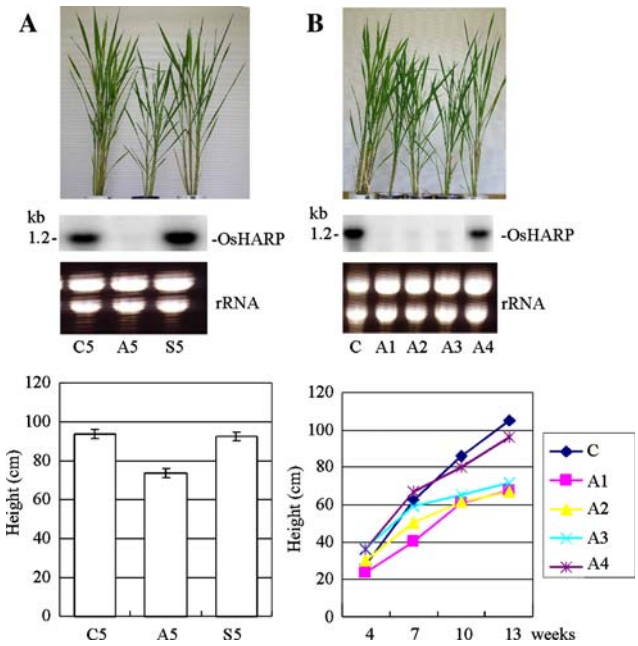

Fig. 6 Phenotypes of sense- and antisense- *OsHARP* transgenic rice. The cDNA of *OsHARP* was introduced into the rice in an antisense or sense orientation under control of the *CaMV35S* promoter in a *pIG121-Hm*. One-month old rice seedlings of vector control, antisense, and sense OsHARP transgenic plant are shown (*upper panel* of **a** and **b**). Expression level of *OsHARP* in transgenic lines was analyzed by Northern blot analysis (*middle panel* of **a** and **b**). Growth of the *OsHARP* sense (S) and antisense (A), and vector control (C) transgenic plants (*lower panel* of **a**), and growth curves of *OsHARP* anti sense transgenic lines (*lower panel* of **b**) were shown

OsHARP antisense transgenic plants show altered growth and development

For *OsHARP* over expression and antisense transgenic rice, the full-length cDNA sequences were amplified by PCR using primer pairs. The *pIG121-Hm/OsHARP* plasmids were then transferred into *Agrobacterium tumefaciens* and transformed into rice. Transgenic rice plants were selected on media containing hygromycin. Plant transformed only with the *pIG121-Hm* vector was used as vector control. The growth of sense *OsHARP* transgenic plants did not change compared with that of vector control. Antisense *OsHARP* transgenic plants were 20–30% shorter than the vector control and sense *OsHARP* transgenic plants at 13 weeks after potting in soil (Fig. 6a). Four *OsHARP* antisense transgenic lines (T2-A1, T2-A2, T2-A3 and T2-A4) were selected for further analysis based on their altered growth. The antisense transgenic line T2-A4 appeared to have developed normally, but was almost 10% shorter. The other three transgenic lines, T2-A1, T2-A2 and T2-A3, were reduced in height by nearly 30% compared with vector control plants (Fig. 6b). To approximate the effect of transcript levels and phenotype, RNA was extracted from the four transgenic lines and compared with the vector control. The three most affected transgenic lines, T2-A1, T2-A2 and T2-A3, had a much reduced level of *OsHARP* transcript, whereas transgenic line T2-A4 more *OsHARP* mRNA than the other three transgenic plants.

The phenotypic changes observed in transgenic plants with repressed *OsHARP* expression further support a role for OsHARP in leaf sheath cell elongation during the vegetative phase of development. Cell elongation is a complex process that requires dramatic changes in the cell walls, including synthesis and enzymatic and non-enzymatic relaxation (Cosgrove 2001; Jan et al. 2004, 2006b; Liszkay et al. 2003). As a result, we examined the downstream components of OsHARP using proteomics technique as following.

Twenty-one proteins are affected by OsHARP

To examine whether OsHARP would have any effect on patterns of protein accumulation or not, antisense

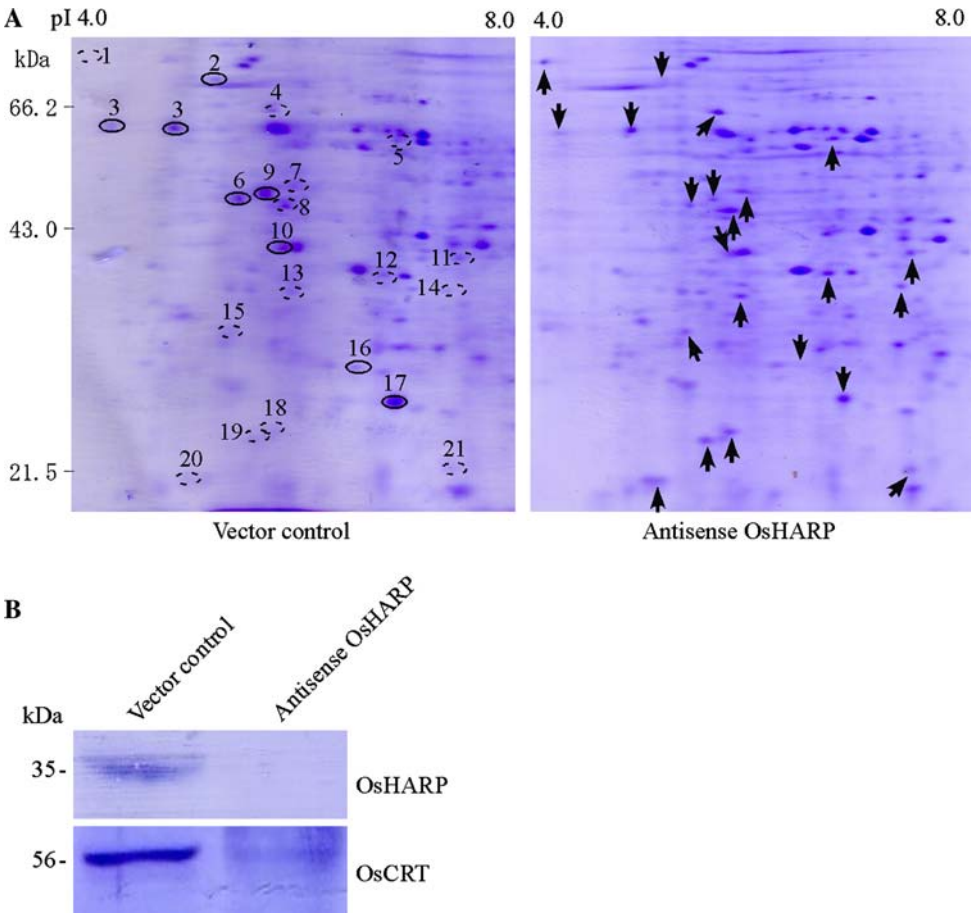

Fig. 7 Protein expression pattern of basal parts of leaf sheath in *OsHARP* antisense transgenic rice. Proteins were extracted from basal parts of leaf sheaths of vector control or *OsHARP* antisense transgenic rice plants. Proteins were separated by 2D-PAGE and stained with CBB (A). *Upward arrows* indicate the positions of up-regulated proteins, *downward arrows* show the position of down-regulated proteins, and *s* represent the same proteins in control. On the other hand, extracted proteins were separated by SDS–PAGE and transferred onto PVDF membrane. Proteins were reacted with anti-OsHARP antibody or anti-OsCRT antibody

transgenic rice plants were analyzed using proteomics. Two-week-old seedlings of antisense *OsHARP* and vector control transgenic rice plants were used. Crude proteins were extracted from basal parts of leaf sheaths, separated by 2D-PAGE, and stained by CBB (Fig. 7). Proteins whose abundance changed in transgenic rice were excised from 2D-gels and identified by protein sequencing or MS (Table 2). Out of 550 proteins, 21 proteins were up-regulated or down-regulated. One of the 21 proteins was OsCRT (spot 3) that must be related to the actions of OsHARP. The functions of 6 of the 21 proteins (spots 1, 9, 11, 15, 17 and 20) were unknown.

Tanaka et al. (2005) reported that the 60S ribosomal protein (spot 19), tubulin (spot 13), enolase (spot 5) and RuBisCO subunit binding-protein (spot 4) were down-regulated during development of the basal part of rice seedlings, but in the antisense-*OsHARP* transgenic rice, these proteins were up-regulated. On the other hand, Tanaka et al. (2005, 2004) and Shen et al. (2003) reported that PP2A (spot 2), heat shock protein (spot 6) and ascorbate peroxidase (spot 16) were up-regulated during development or GA treatment, and these proteins were down-regulated in antisense-*OsHARP* transgenic rice. These results indicate that OsHARP is affected by development of the basal part of leaf sheaths and by GA, and somehow these coordinately regulated proteins are also related to OsHARP.

Concluding remarks

Shen et al. (2003) reported that studies on proteomics of rice leaf sheath tissue showed a total of 352 proteins separated on a 2D-PAGE, and GA_3 showed 21 and 11 proteins as up-regulated and down-regulated, respectively, representing nearly 10% of the proteins modulated during leaf-sheath elongation. They also identified that of the 32 predicted proteins modulated by GA_3 response, homologies for two protein spots were found to be OsCRT by protein sequencing (Shen et al. 2003). Though, the Mr of these twin spots was 56 kDa, the same as that of OsCRT as demonstrated earlier by Komatsu et al. (1996), the difference in the pI between two proteins was 4.3 and 4.0, respectively. However, in the presence of growth inhibitor, uniconazole and ABA, no elongation of the leaf sheath or shift in OsCRT from the basic site to

Table 2 Characterization of changed proteins in antisense OsHARP transgenic rice

Spot no.	N-terminal sequences	Homologous protein (homology %)	Accession no.	Ratio
1	SDPLFYEPFD	Functional unknown (100)	AL606610	11.0 ± 3.6
2	Blocked (MS)	PP2A regulatory subunit	AY224436	0.4 ± 0.1
3	EVFFQEKFED	CRT (100)	Q9SP22	0.3 ± 0.1
4	AAKDIAFDQH	RuBisCO subunit binding-protein alpha subunit (100)	P08926	6.3 ± 1.5
5	Blocked (MS)	Enolase	AY335488	3.0 ± 1.0
6	STAQTTIEID	dnaK-type molecular chaperone hsp70 (100)	S48024	0.6 ± 0.2
7	Blocked (MS)	Actin	X15865	2.2 ± 0.8
8	Blocked (MS)	Adenosine kinase	AB05624	10.0 ± 2.0
9	TAXEPAKRTL	Functional unknown (100)	AB3003	0.7 ± 0.2
10	Blocked (MS)	Glycine-rich RNA-binding protein	AJ002893	0.7 ± 0.1
11	Blocked (MS)	Functional unknown	AP003933	4.6 ± 2.1
12	Blocked (MS)	Cysteine synthase	AF07697	3.3 ± 1.5
13	Blocked (MS)	Tubulin	X78142	3.7 ± 1.5
14	MLDIPPATES	Malate dehydrogenase (100)	Q7XDC8	2.7 ± 1.2
15	QAPALAA	Functional unknown (100)	AP005605	3.7 ± 2.1
16	ALIAEKSCA	L-Ascorbate peroxidase 1, cytosolic (100)	P93404	0.8 ± 0.1
17	AVEEVKK	Functional unknown (100)	Q7JJZ	0.5 ± 0.2
18	AARAVKETT	NADH-ubiquinone oxidoreductase (100)	AE010303	4.0 ± 1.0
19	ATAAEKKVV	60S ribosomal protein L17-A (100)	P05740	5.0 ± 1.0
20	GVYTFVXRSS	Functional unknown (100)	AP003726	8.0 ± 2.0
21	ATKKVAVLK	V-type ATP synthase subunit E (89)	Q57673	4.7 ± 2.5

The ratio is "protein expression volume in antisense OsHARP transgenic rice/protein expression volume in vector control"

The ratios are the means ± SE of three independent experiment sets

acidic were observed. These results suggested that OsCRT is an important component in GA signaling that regulates rice seedling leaf sheath elongation.

Based on previous reports and these results, OsCRT is sharing multiple functions in rice. The identification of OsCRT as a phosphoprotein during the regeneration of rice cultured suspension cells suggests a role in differentiation and regeneration processes. Furthermore, the screening of OsCRT-interacting proteins through yeast two-hybrid analysis identified a new family of putative regulatory proteins in plants typified by the ubiquitin domain (*OsCRTintP1*; Sharma et al. 2004) and having a high number of histidine and alanine residues (OsHARP), that play a role in controlling stress-responsive proteins (Komatsu et al. 2007) and leaf sheath elongation indicated in this research, respectively. These results signify an important role of the CRT-CRTintP1 complex in rice stress response and CRT-HARP complex in rice leaf sheath elongation. The next step toward understanding OsHARP is to clarify to determine whether the reduction in plant height results from defect in leaf sheath cell elongation.

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